The Interaction of GIGANTEA with the LOV Domain of ZEITLUPE

A Senior Honors Thesis

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Introduction

The plant circadian clock is a complex biological mechanism by which the plant is able to sense environmental stimuli, entrain itself to those environmental conditions, and predict future environmental conditions. The circadian clock functions with a roughly 24 hour rhythm, responding to the diurnal light and temperature cycles of the 24 hour day. The clock has three major components: the input pathway, the central oscillator, and the output pathway. The input pathway is the mechanism by which the plant senses and responds to the environment. The central oscillator is a system of protein interactions, gene regulation, and interlocking feedback loops that establishes a self sustaining 24 hour rhythm and then controls the output pathways. The output pathways of the circadian clock are the functions of the plant that are controlled by the clock including leaf movement, hypocotyls growth, flowering time and gene expression[1].

The central oscillator is of major interest currently in the area of circadian clock research. In the past decade many important plant clock proteins and pathways have begun to be discovered. Using Arabidopsis as the model organism for studying the plant circadian clock, a network of three major interlocking negative feedback loops has been discovered[1]. In addition to these three negative feedback loops, there is a fourth loop, referred to as Loop D that infers light-sensitive post-translational regulation onto loop B of the clock thereby affecting the entire plant circadian clock[1]. This loop D is the focus of the work conducted in this report.

Loop D currently consists of five proteins, ZEITLUPE (ZTL), TIMING OF CAB EXPRESSION 1 (TOC1), GIGANTEA (GI), PSEUDO RESPONSE REGULATOR 3 (PRR3), and PSEUDO RESPONSE REGULATOR 5 (PRR5). ZTL protein has three

domains, a Kelch repeat domain, an F-box domain, and a LOV domain (light, oxygen, or voltage sensing domain). As an F-box protein, ZTL forms a complex with components of E3 ligase of the SCF complex and targets proteins for ubiquitin mediated degradation via the 26S proteasome pathway[2]. Of primary interest to us is ZTL's role in targeting TOC1 for degradation, TOC1 being a component in the negative feedback Loop B. ZTL is stabilized by GI via a posttranslational interaction that is increased 3-fold in blue light[2]. The following report deals specifically with the interaction of GI with ZTL.

In previous unpublished research by Ruishuang Geng and David Somers, the LOV (ZTL amino acids 1-192) and LOV-F (ZTL amino acids 1-280) domains of ZTL, driven by 35S promoters were expressed in Arabidopsis. This was conducted to further investigate ZTL action and specifically the role that the LOV domain may play. These transgenic lines showed interesting phenotypes that encouraged further investigation into the role that the LOV domain may play. The first phenotype observed was period lengthening. The transgenic lines over expressing the LOV and LOV-F domains also express the clock reporter gene, *luciferase*, driven by the clock controlled CAB2 promoter[2]. Supplemental data Figure 1 shows how the transgenic lines show period lengthening of 1-2.5 hours in both constant red and constant blue light. The period lengthening was investigated by analyzing endogenous ZTL levels. The top and bottom panels of Supplemental Figure 2 show that there was a significant reduction in endogenous ZTL levels in each of the transgenic lines. The middle panel of Supplemental Figure 2 shows that this reduction in ZTL is not due to down regulation at the transcriptional level because there is no change in mRNA levels in the transgenic lines. The second important observable phenotype was an increase in flowering time. The

transgenic lines were grown under long and short days and under long days there was a significant increase in flowering time, measured by increased number of rosette leaves before bolting[4]. These results can be found in Supplemental Figure 3. These observed phenotypes provoked further inquiry into the cause of the phenotypes by analyzing ZTL and LOV domain interactions with a well known clock and flowering time related gene, *GIGANTEA*[5-7].

Here we use the LOV over expressing line (LOV OX) to investigate the ZTL interaction with GI. We show that the interaction between GI and ZTL occurs at the LOV domain, and that this interaction competes with the interaction between GI and endogenous ZTL in the GI-HA with LOV OX background. We show that there exists a co-stabilizing effect of GI on ZTL[2] and ZTL on GI (shown here) by showing an increase in GI when LOV OX is present, and we confirm that this stabilization is post-translational.

Results

The LOV Domain of ZTL interacts with GI

To determine if GI interacts specifically with the LOV domain of ZTL a co-immunoprecipitation was performed. The protein was extracted from the tissue of 10 day old seedlings, stably expressing LOV OX with GI-HA, LOV OX alone, and GI-HA alone and were collected at ZT 13 when GI expression is highest. Prior to the protein extraction, a crosslinking procedure was conducted to ensure that transient interactions would be detectable in the co-immunoprecipication. Significant amounts of GI could be immunoprecipitated using the HA antibody in the tissue samples that contained GI-HA

(Figure 1; lanes 4-5). Then when probed with a ZTL anti-body that detects the LOV domain, the LOV domain was detected only in the extract from the tissue containing LOVOX and GI-HA (Figure 1, lane 5) thus indicating that LOV had been successfully co-IPed by GI using an HA antibody. (Figure 1)

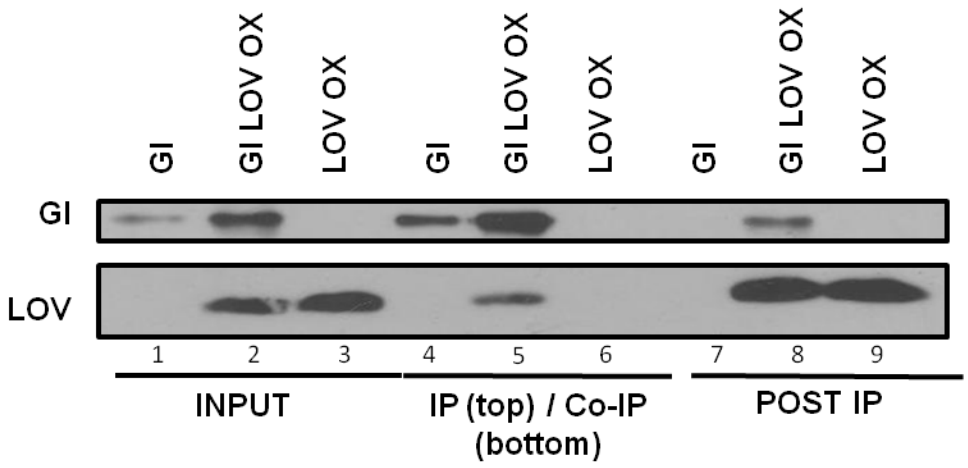


Figure1: GI LOV-OX, LOV Co-IP

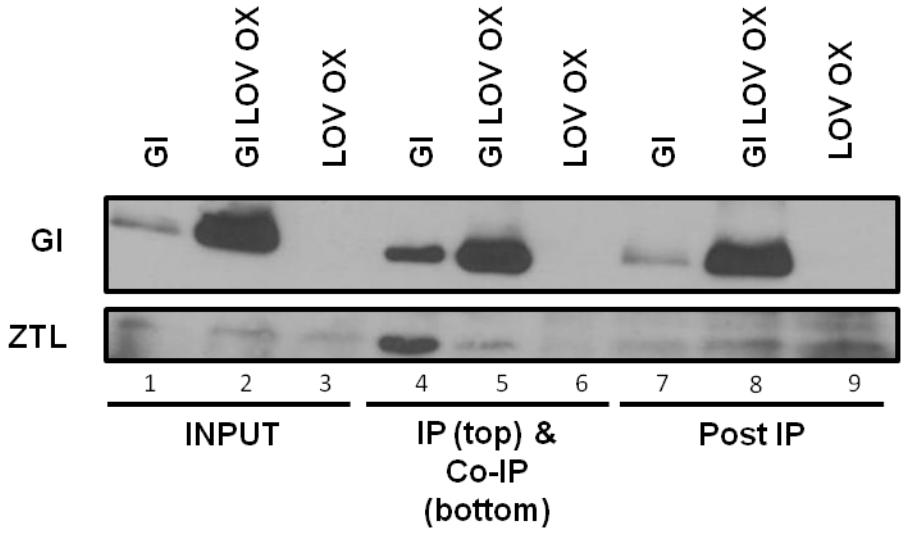
Protein was extracted from tissue expressing GI-HA alone, GI-HA and LOVOX, and LOVOX alone. The input columns (lanes 1-3) show total protein extracts probed with anti-HA (top membrane) and anti-ZTL (bottom membrane) and show that GI-HA is present in all lines except LOV alone (by observing the 120kDa GI band) and that LOV is present in each line (by observing the roughly 20 kDa ZTL band) except in GI-HA alone. The IP and Co-IP columns (lanes 4-6) show that GI was successfully IPed in the primary IP using anti-HA in all lines except LOVOX alone. The columns also show that LOV was successfully pulled down by GI in the GI-HA and LOVOX background and the bands were observed by probing with anti-ZTL antibody. The Post IP columns (lanes 7-9) show the protein extract after the incubation of the extract with the resin bound primary anti-HA anti-body. Lane 8 shows that unbound GI remains after the incubation with the GI and LOVOX background and lanes 8 and 9 show that unbound LOV remain after the incubation. Data shown is representative of two independent trials.

The Interaction of GI and LOV can explain the reduction of Endogenous ZTL

In LOV OX transgenic lines, endogenous ZTL levels are reduced considerably and this is believed to contribute to the lengthening of the period observed in the LOV OX lines. To determine whether it is the interaction between LOV and GI that is causing

this reduction of endogenous ZTL, co-immunoprecipitations were again performed using GI to pull down endogenous ZTL in the GI-HA and GI-HA with LOVOX backgrounds. The results are shown in Figure 2 a. Figure 2 a lanes 4 and 5 show that endogenous ZTL can be pulled down by GI in both backgrounds yet by comparing lanes 4 and 5, we observe greater relative amounts of ZTL associating with GI in the GI-HA alone background (lane 4) than in the GI-HA and LOVOX background (lane 5). There is significantly less ZTL in GI-HA immunoprecipitates when LOVOX is present. The presence of ZTL in the post-IP fraction (Figure 2 a lane 8) shows that this reduction in ZTL is not due to a net reduction in ZTL present. Figure 2 b shows a quantification of the results found in Figure 2a. The quantification is a ratio of the amount of ZTL pulled down to the amount of GI IP-ed in the two backgrounds. The ratios clearly show a significant reduction in the amount of ZTL pulled down by GI when LOV OX is expressed.

a)



b)

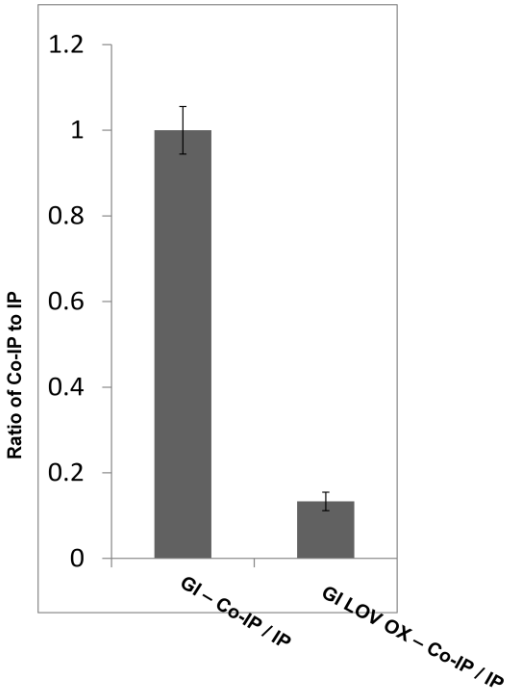


Figure 2: GI and GI LOVOX ZTL Co-IP

a) Protein was extracted from tissue expressing GI-HA alone, GI-HA and LOVOX, and LOVOX alone. The input columns (lanes 1-3) show total protein extracts probed with anti-HA (top membrane) and anti-ZTL (bottom membrane) and show that GI-HA is present in all lines except LOV alone (by observing the 120kDa GI band) and that ZTL is present in each line (by observing the roughly 75 kDa ZTL band). The IP and Co-IP columns (lanes 4-6) show that GI was successfully IPed in the primary IP using anti-HA in all line except LOVOX alone. The columns also show that ZTL was successfully pulled down by GI in the GI-HA alone background and the GI-HA and LOVOX background and the bands were observed by probing with anti-ZTL antibody. The Post IP columns (lanes 7-9) show the protein extract after the incubation of the extract with the resin bound primary anti-HA anti-body. Lanes 7 and 8 show that unbound GI remain after the incubation and lanes 7-9 show that unbound ZTL remain after the incubation. Data shown is representative of two independent trials.
b) The bands for Co-IPed ZTL in figure 2 (a) lanes 4 and 5 were quantified and were divided by the quantified GI bands in lanes 4 and 5. These ratios are displayed in this figure showing that the ratio of ZTL pulled down by GI in lane 4 is far larger than the ratio of ZTL pulled down by GI in lane 5.

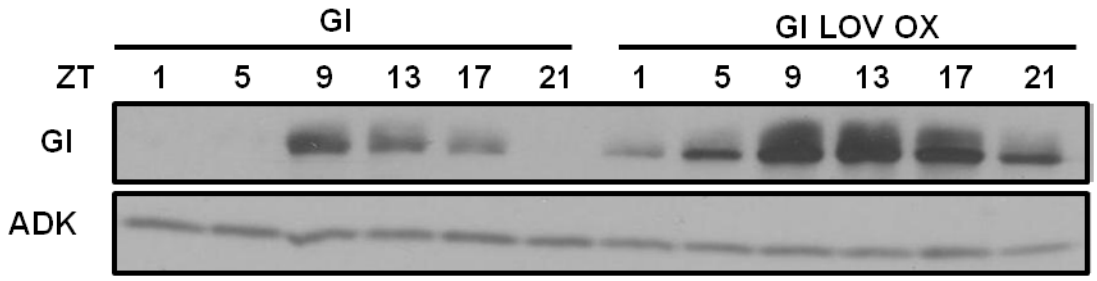
The LOV domain Stabilizes GI

Tissue expressing GI-HA alone and GI-HA with LOVOX was collected every 4 hours over a light/dark cycle. A western blot was performed on each of these tissue

samples on the same gel to analyze any difference in GI protein levels between the two genotypes. Figure 3 a shows that there was a clear accumulation of GI protein in the LOV OX background when compared to the WT background.

To determine whether this accumulation of GI protein was due to post-translational stabilization, q-PCR was performed and shows that the GI mRNA -levels remained unchanged in the two backgrounds. The q-PCR results found in Figure 3 b confirmed that there were insignificant changes in GI mRNA expression between the LOVOX with GI-HA line and the GI-HA line.

a)



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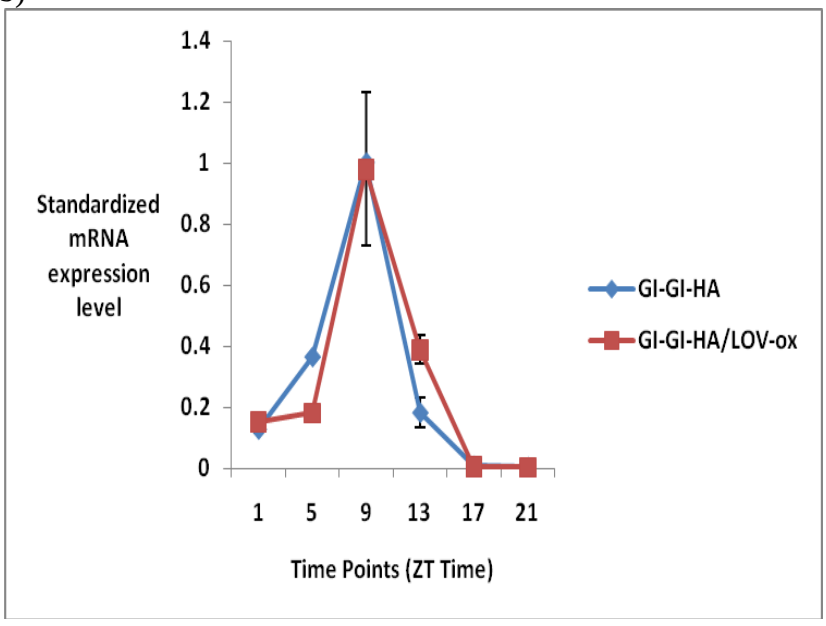

Figure 3: Post-translational Stabilization of GI by LOV

a) Protein was extracted from GI-HA alone and GI-HA with LOVOX tissue for timepoints ZT1, 5, 9, 13,17, 21 and these protein extracted were run on a single protein gel. The membrane was probed with anti-HA antibody to observe GI levels in the two backgrounds (top membrane). The membrane was also probed with anti-ADK to use as a loading control to show equal loading (bottom membrane). This time series western blot was conducted twice with identical results. Data shown is representative of two independent trials.
b) *GI* mRNA levels were analyzed in the GI-HA alone and LOVOX backgrounds using q-PCR. The q-PCR shows very little difference in *GI* expression in the LOVOX transgenic background. This q-PCR was conducted twice with similar results and the error bars represent standard error of the mean.

Discussion

ZTL is well known as an essential component of the plant circadian clock as the protein responsible for degradation of TOC1 in a circadian manner. GI also is known as an important clock regulated protein that imposes a circadian rhythm onto ZTL via its light regulated stabilization of ZTL. Here we confirm and extend the original observation[3] that the ZTL interacts with GI via the LOV domain of the ZTL protein. The results clearly show that GI can interact and bind with the independent LOV domain, demonstrating that the LOV domain is sufficient for the ZTL and GI interaction. To demonstrate that this interaction was present *in vivo*, crosslinking was necessary prior to the extraction of the protein from the Arabidopsis tissue. This interaction between GI and LOV *in vivo* must be short lived, thus requiring crosslinking to preserve the interaction through the protein extraction process.

GI was also used to pull down endogenous ZTL in the GI-HA alone line and the GI-HA with LOVOX line to compare the ability of GI to pull down ZTL in these backgrounds. This experiment was necessary to investigate whether the presence of the LOV domain diminished the amount of endogenous ZTL pulled down by GI. The results clearly show that when the LOV domain is expressed, less endogenous ZTL associates with GI, demonstrating that there is a decreased interaction between these two

compounds. We conclude that the decreased interaction is likely due to the presence of high levels of LOV polypeptide, sequestering the available GI into GI – LOV complexes, therefore leaving less free GI available to bind to ZTL. This would then cause a decrease in endogenous ZTL levels due to the loss of the stabilizing GI – ZTL interaction. Therefore, it is the GI – LOV interaction that causes the reduction in endogenous ZTL. The presence of unbound ZTL in the post-IP sample demonstrates that the reduced ZTL pulled down by GI in the LOVOX background is not due to limited availability of ZTL resulting from the reduced ZTL levels in that background (Supp. Figure 2).

The notion that the abundant LOV domain sequesters GI, also can be used to explain the lengthened flowering time. GI is involved in stabilizing FKF1, which up regulates CO expression through the removal of the CDF repressors[8]. This increase in CO activates FT which promotes flowering[9-10]. If GI is sequestered by LOV it becomes unavailable to serve its function of stabilizing FKF1, *CO* expression remains repressed and therefore flowering time is delayed.

The stabilization of ZTL by GI was reported by Kim et al. (2007) but our work here demonstrates that ZTL must also play an essential role in stabilizing GI. This stabilization was demonstrated by showing that the total GI protein expressed in the GI-HA with LOVOX line far exceeds the GI levels in the GI-HA alone background at each of 6 time points. q-PCR confirms that this increase in GI protein levels is not due to a corresponding increase in GI mRNA expression and therefore we can conclude that the increase is to due to post-translational stabilization. By demonstrating that the LOV domain stabilized GI, we can also conclude that ZTL must co-stabilize GI via the ZTL – GI interaction. The stabilization of ZTL by GI helps explain why ZTL has a protein

oscillation, how it is able to drive greater TOC1 oscillations, and infer some light sensitivity onto the clock. But the reverse stabilization may play a role in regulating GI's functions. GI is a very versatile protein that contributes to the clock as well as to the flowering time regulation pathway, and its stabilization by ZTL may confer some regulation onto flowering time. ZTL may stabilize GI and help drive increased GI oscillation by allowing it to accumulate to the higher levels needed for proper flowering time regulation. These two results of the interaction would provide one mechanism of light regulation of flowering time (the blue light dependence of the ZTL and GI interaction would help regulate GI protein levels) and further explain clock regulation of flowering time (by providing additional information about the mechanism by which a clock controlled protein, GI, can regulate flowering time). The theory that ZTL helps GI accumulate to higher levels is also substantiated by Figure 1c from Kim et al. (2007) where GI protein levels are shown to decrease and oscillation of GI decreases in the *ztl* background.

Materials and Methods:

Cross-linking: Arabidopsis seedlings are grown for 10 days and then harvested. The seedlings are soaked in 20mL of ice-cold 1x PBS (Phosphate Buffered Saline). Formaldehyde is added to make the total concentration 1% formaldehyde and then mixed by inverting. The seedlings in the PBS and formaldehyde solution is vacuum infiltrated for 30 minutes in an ice bath. Ice cold glycine is added to bring the final concentration to 300mM and then the vacuum infiltration is continued for 30 minutes. Following the

infiltration, the seedlings are washed three times in 1x PBS and then frozen in liquid nitrogen.

Protein extraction: Protein extraction were performed according to ref. 3.

Co-immunoprecipitation: Immunoprecipitation was performed according to ref. 3, with minor modifications. The anti-body used was raised against the HA tag instead of the GFP tag that was used in ref.3

Western Blot: immuneblotting was performed according to ref.3

RNA extraction: 1mL of Trizol Reagent (Invitrogen) was added to 200uL of frozen ground tissue and the tissue was allowed to dissolve at room temperature for 5 minutes. 200uL of chlorophorm was added to tubes and the tubes were then shaken for 15 seconds and then allowed to sit for 2 minutes at room temperature. The tubes were then centrifuged for 15 minutes at 11,600 rpm. Aqueous upper layer from the tubes were then alequated to new tubes and 500uL of isopropanol was added. The tubes were then allowed to incubate at room temperature for 10 minutes followed by 10 minutes of centrifugation at 14,000 rpm. The pellet was then washed with 75% ethanol and then centrifuged for 5 minutes are 7,500 rpm. The pellet was then re-suspended in DEPC (Diethylpyrocarbonate) water. The RNA was quantified using the Nanodrop spectrophotometer ND-1000 (Thermo Scientific) and 12ug of RNA is transferred to new tubes for DNase treatment.

DNase treatment: DNase reagents were included in kit from Ambion. 12ug of RNA is brought to a total volume of 15uL. 2.5uL of 10x DNase I buffer and .3uL of DNase I were added to the RNA and incubated at 37°C for 30 minutes. DNase

inactivation beads (1/10th the total volume) were then added and allowed to settle. The supernatant was then alequated into a new tube.

RT-PCR: RNA, suspended in DEPC water was incubated at 65°C for 10 minutes and cooled them down on ice for 5 minutes. 1µg of RNA is added to a reverse transcription mix that includes: 1µL Oligo(dT) (0.5µg/µL), 4.0 µL ImProm-II 5X reaction buffer, 4.0 µL $MgCl_2$ (25mM), 1.0µL dNTP mix (10mM), 1.0µL ImProm-II Reverse Transcriptase, 8 µL Nuclease-Free water. This creates a final volume 20.0µL. The reaction was incubated at 25°C for 10 minutes followed by incubation at 42°C for 60 minutes, then 99°C for 5 minutes, and finally 4°C for 5 minutes.

q-PCR: Reaction reagents were included in SuperScript III kit from Invitrogen. The reaction mixture for the q-PCR was comprised of 7.5 µL 2X RT-PCR buffer, 1.5 µL primer set, 3 µL cDNA, and 3 µL DEPC water for a final volume of 15 µL. The experiment included two technical trials for each time point under investigation (ZT 1,5,9,13,17,21) for each phenotype under investigation (GI-HA alone and GI-HA with LOVOX) using the primer sets for gigantean (forward primer: AATTCAGCACGCGCCTATTG, reverse primer: GTTGCTTCTGCTGCAGGAACTT). The experiment also included a single technical trials for each time point under investigation for each phenotype under investigation using the primer sets for actin (forward primer: CAGTGTCTGGATCGGAGGAT, reverse primer: TGAACAATCGATGGACCTGA), to act as a non-oscillating control that was used to standardized the measurements of GI message levels at each time point. The experiment also included two trials of the serial dilutions of the cDNA. 10 µL of the cDNA from GI-HA LOVOX ZT 13 was first mixed with 10 µL of DEPC water then 10 µL of this

dilution was mixed with 10 µL of DEPC water and this dilution pattern was repeated 3 more times to generate a serials of dilutions of the ZT 13 cDNA (1X, ½X, 1/4X, 1/8X, 1/16X, 1/32X). The experiment also included negative controls for both primer sets, the reaction mixture without the cDNA included. The reaction protocol for the q-PCR reaction proceeded as follows: 94°C for 2 minutes, followed by 40 cycles of 94°C for 15 seconds and 60°C for seconds. The experiment was conducted using the facilities at the Plant-Microbe Genomics Facility at The Ohio State University.

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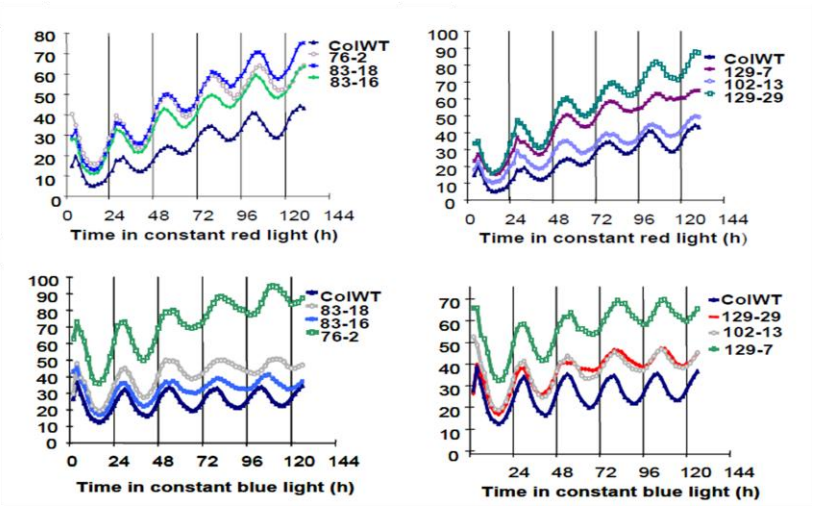
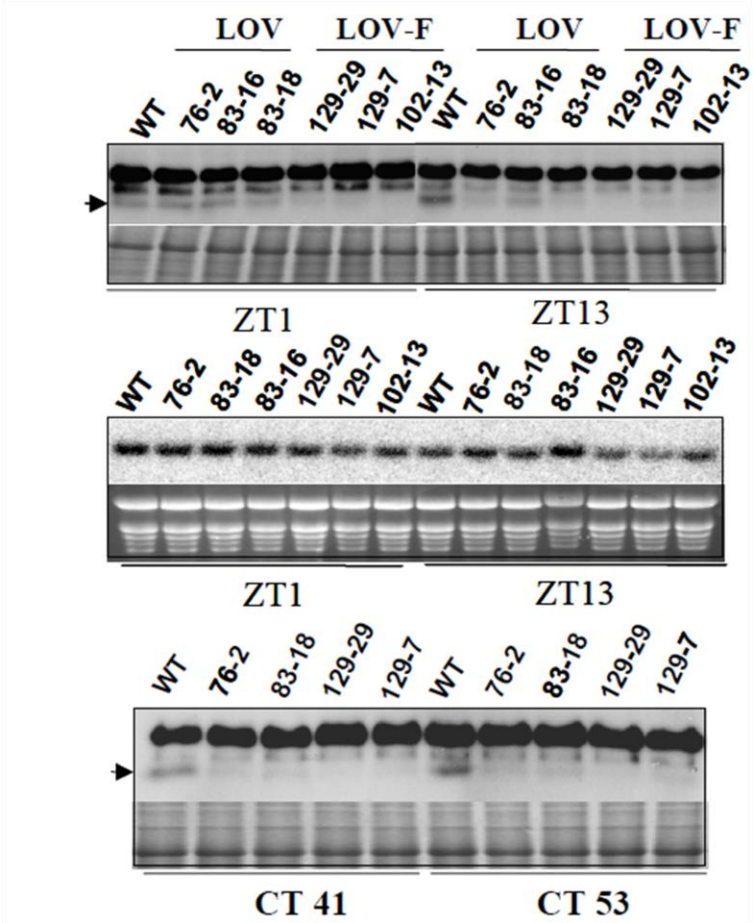
Supplemental Data:
Data can be found in Reference 3



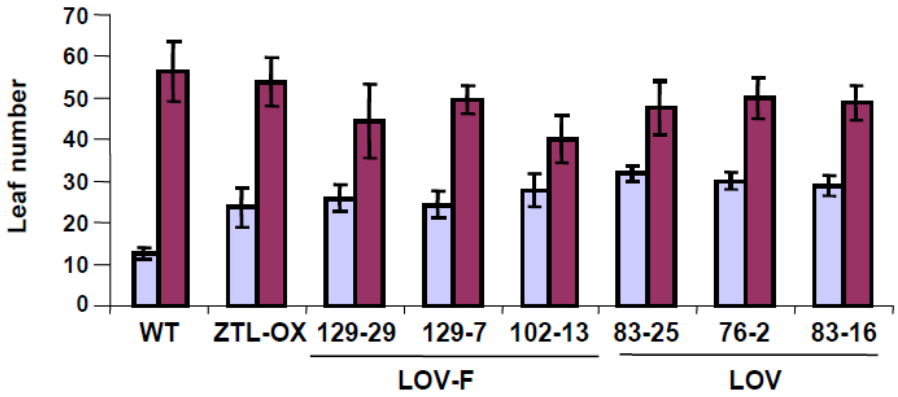
Supplemental Figure 1: LOV and LOV-F expression lengthens free-running period

The clock period was analyzed by observing the oscillations of the luciferase expression in the LOV and LOV-F backgrounds and are compared to the expression in the WT background in both constant red light and constant blue light. Three lines from LOV (76-2, 83-16, 83-18) and three LOV-F lines (129-29, 129-7, 102-13) are shown.



Supplemental Figure 2: Posttranscriptional reduction of endogenous ZTL protein in LOV and LOV-F expressing plants.

Endogenous ZTL levels were analyzed by running western blots on WT tissues as well as each of the LOV and LOV-F expression lines (lines are as in figure 1) collected at ZT 1 and ZT 13 and probing for ZTL with an anti-ZTL anti-body. The top figure shows ZTL levels from seedlings grown in 12 hours light and dark cycles with the lower panel showing the coomassie stain loading control. The middle figure shows ZTL mRNA expression level with the lower panel showing total RNA loaded as a loading control. The lowest figure shows ZTL protein levels in seedlings entrained in 12 hour day and night cycles yet were placed in constant blue light for 3 days before harvest. The lower panel shows a coomassie stain loading control. The black arrows in this figure indicate the ZTL band. CT=circadian time (clock time of the organism, ratio of the free running time to 24 hours), ZT=Zeitgeber Time



Supplemental Figure 3: LOV and LOV-F expression delays flowering time in long days

The number of leaves (rosette and cauline) of WT, ZTL overexpressor, LOV and LOV-F overexpressors at flowering under long days (light fill) and short days (dark fill)